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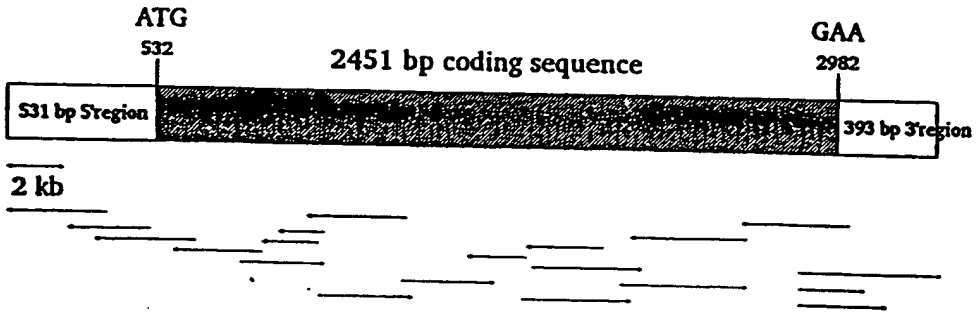

(57) Abstract

This invention relates to improved fungal cells and methods for producing recombinant products of improved quality and in high yields. More specifically, the present invention relates to fungal cells carrying specific modifications within their DNA sequences which cause them to exhibit at least a reduced capacity for O-glycosylating homologous and/or heterologous proteins, and the use of these cells as host cells to produce high yields of recombinant products.

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MODIFIED FUNGAL CELLS AND METHOD FOR PRODUCING RECOMBINANT PRODUCTS

This invention relates to improved fungal cells and methods for producing recombinant products of improved quality and in high yields. More specifically, the present invention relates to fungal cells carrying specific modifications within their DNA sequences which cause them to exhibit at least a reduced capacity for O-glycosylating homologous and/or heterologous proteins, and the use of these cells as host cells to produce high yields of recombinant products.

The development of recombinant DNA technology has made possible the production of foreign products in host cells in which exogenous DNA sequences coding for those products have been introduced. The advantage of this technology is that products can be produced in high yields, in highly purified form, with no risk of contamination such as viral contamination (AIDS, hepatitis B, etc.). These recombinant techniques have been widely used for the production of recombinant proteins in prokaryotic as well as eukaryotic host cells. Prokaryotic host cells include *E. coli* [Nagata et al., Nature 284 (1980), 316; EP 001 929], *Bacillus subtilis* [Saunders et al., J. Bacteriol. 169 (1987), 2917], *Streptomyces*, and *Corynebacterium* (EP 433 117). Eukaryotic host cells include plant cells, animal cells and fungal cells.

However, the large-scale production of recombinant products by these techniques is still limited, due to problems of expression efficiency of these exogenous DNA sequences, due also to vector instability and to intracellular degradation of the recombinant products by the host cell in which they are made. Concerning expression efficiency, efforts have been made to isolate strong promoters, leading to increased expression levels of exogenous DNA sequences, and therefore to increased production levels of recombinant products. Various systems have also been developed in order to increase the stability of the vectors within the host cells, the most frequently used of which consisting in the

insertion on the vector of an antibiotic resistance gene enabling recombinant host cells to survive and grow in a selective medium. With respect to intracellular degradation, several mutant cells lacking or having a reduced protease activity have been disclosed, thereby limiting the capacity of said cells to degrade recombinant products.

However, additional problems still limit large-scale production and pharmaceutical use of recombinant products. One of these arises from the fact that recombinantly produced products are often different from their natural counterparts. For example, bacterial host cells do not possess all the post-translational mechanisms required for maturation of mammalian polypeptides. Accordingly, said mammalian polypeptides produced in bacteria are often immature or not correctly refolded. Furthermore, bacterial host cells generally introduce an additional N-terminal methionine to the products.

Recombinant products produced in heterologous eukaryotic hosts also usually differ from their naturally-occurring counterpart in their glycosylation content. This may concern the presence versus absence of any carbohydrate structure, the localization of said carbohydrate structure on the product, as well as the nature of the carbohydrate. More specifically, it has been shown that yeast-derived recombinant products often bear additional unnatural O-glycans compared to their natural counterpart. For instance, it has been shown that, while human serum insulin-like growth factor I (IGF-I) is not glycosylated, its recombinant form produced in *S. cerevisiae* is O-glycosylated and, more precisely, O-mannosylated [Hard et al., FEBS Letters 248 (1989), 111]. In the same way, it has been shown that human platelet-derived growth factor (PDGF) and human GM-CSF display unnatural O-mannosyl structures when produced in *S. cerevisiae* [Biomedic. Environ. Mass Spectrometry 19 (1990), 665; BIO/TECHNOLOGY 5 (1987), 831]. This abnormal O-glycosylation is the result of important differences between the glycosylation mechanisms of mammalian (human) cells and those of other eukaryotic cells, such as yeasts. In this respect, it has been observed that O-glyco-

sylation in fungal cells (including yeasts and filamentous fungi) proceeds in a similar and unusual way so far not observed in any other organism.

The occurrence of this undesirable O-glycosylation on fungal-derived recombinant products constitutes an important drawback to this technology for the production of pharmaceuticals.

The first reason is that fungal-specific glycans may introduce new immunological determinants on a protein, and a glycoprotein with such unnatural carbohydrates may therefore be antigenic when administered to humans. In this respect, it is known for example that most humans have antibodies directed against N-linked yeast mannan chains [Feizi and Childs, Biochem. J. 245 (1987), 1].

Another reason is that proteins without appropriate carbohydrate structures may also have altered pharmacokinetic properties. It has been shown that carbohydrate structures of glycoproteins influence and participate in defining their *in vivo* clearance rate, which is essential in determining the efficacy of a pharmaceutical. More precisely, a mannose receptor has been identified on the surface of liver endothelial cells and resident macrophages which apparently represents a means for eliminating glycoproteins displaying mannose-type oligosaccharides [Stahl, Cell. Mol. Biol. 2 (1990), 317]. Therefore, the presence of unnatural, additional mannose structures on a protein may increas its clearance rate and thus decrease its plasma half life.

Still another reason is that biological activity of a glycoprotein has also been shown to vary with its carbohydrate content, location and nature. For example, it has been shown that glycosylation affects the biological properties of recombinant human EPO [Takeuchi et al., Proc. Natl. Acad. Sci. USA 86 (1989), 7819] and recombinant human tPA [Parekh et al., Biochemistry 28 (1989), 7644].

For the reasons mentioned above, it is clear that the unnatural O-glycosylation of fungal-derived recombinant products can severely affect their immunological, biological and pharmacokinetic properties, and therefore may prevent their development for human therapeutic use.

The present invention solves the problem of abnormal O-glycosylation referred to above by providing modified fungal cells carrying genetic modification(s) within their DNA sequences which cause them to have at least a reduced capacity for O-glycosylating native or foreign proteins.

Applicant has found that it is possible to obtain genetically modified fungal cells having reduced capacity of O-glycosylation which are still viable and show good growth characteristics in industrial fermentation conditions. Unexpectedly, Applicant has also shown that said genetic modifications do not affect stability of these fungal cells when transformed with exogenous DNA. The modified fungal cells of the present invention can be utilized advantageously as host cells for the production of recombinant products of high quality, having reduced or no undesirable O-glycans.

One object of the present invention is a fungal cell carrying genetic modification(s) within its DNA sequences which cause it to have at least a reduced capacity of O-glycosylation.

The fungal cell of the present invention can be chosen from filamentous fungi and yeasts. Exemplary genera of filamentous fungi contemplated by the present invention are *Aspergillus*, *Trichoderma*, *Mucor*, *Neurospora*, *Fusarium* and the like. Exemplary genera of yeasts include *Kluyveromyces*, *Saccharomyces*, *Pichia*, *Hansenula*, *Candida*, *Schizosaccharomyces* and the like. More preferred genera are those selected from the group consisting of *Kluyveromyces*, *Saccharomyces*, *Pichia*, *Hansenula* and *Candida*, and, even more preferred, from *Kluyveromyces* and *Saccharomyces*. Exemplary strains of *Kluyveromyces* which constitute preferred embodiments of this invention include *K. lactis*, *K. fragilis*, *K.*

waltii, *K. drosophilarum* and the like. The preferred strain of *Saccharomyces is S. cerevisiae*.

In the meaning of the present invention, genetic modification preferably means any suppression, substitution, deletion or addition of one or more bases or of a fragment of the fungal cell DNA sequences. Such genetic modifications may be obtained *in vitro* (directly on isolated DNA) or *in situ*, for example by genetic engineering techniques or by exposing the fungal cells to mutagenic agents. Mutagenic agents include for example physical agents such as energetic rays (X-rays, χ-rays, UV, etc.) or chemical agents capable of reacting with different functional groups of DNA, such as alkylating agents (EMS, NQO, etc.) bisalkylating agents, intercalating agents, etc. Genetic modifications may also be obtained by genetic disruption, for example according to the method disclosed by Rothstein et al. [Meth. Enzymol. 194 (1991), 281-301].

According to this method, part or all of a gene is replaced, through homologous recombination, by an *in vitro* modified version.

Genetic modifications can also be obtained by any mutational insertion on DNA sequences, such as transposons, phages, etc.

In addition, it is known that certain modifications such as point mutations can be reversed or attenuated by cellular mechanisms. Such modifications may not provide the most useful forms of modified fungal cells of this invention since their phenotypical properties may not be very stable. The present invention also provides a process for preparing modified fungal cells in which the modifications (and therefore the phenotypical properties) are stable during segregation and/or non-reverting and/or non-leaky. Such modified fungal cells are particularly advantageous as hosts for the production of recombinant products.

Accordingly, a preferred embodiment of the invention is a fungal cell carrying genetic modification(s) which are stable during

segregation and/or non-reverting and/or non-leaky. These modifications are generally obtained by deletion(s) or disruption(s).

The genetic modification(s) carried by the fungal cells of the invention can be located either in a coding region of the DNA sequences of the cell or in a region responsible for or involved in the expression and/or transcriptional regulation of a gene. More particularly, said modification(s) will generally affect the coding region or the region responsible for or involved in the expression and/or the transcriptional regulation of one or more genes whose expression products are enzymes of the O-glycosylation pathway.

The reduced capacity of the fungal cells of the invention to O-glycosylate proteins may therefore result from the production of inactive enzymes due to structural and/or conformational changes, from the production of enzymes having altered biological properties, from the absence of production of said enzymes, or from the production of said enzymes at low levels.

The fungal cell O-glycosylation pathway involves attachment of a first mannosyl residue to the hydroxyl group of seryl and/or threonyl amino acids of proteins or peptides, and then the extension to O-linked di- and oligosaccharides by subsequent addition of mannosyl residues. The first mannosyl residue is transferred from dolichol monophosphate mannose (Dol-P-Man) to the protein in the endoplasmic reticulum, and the additional mannosyl residues are transferred from GPD-Man in the Golgi. In contrast, higher eukaryotic (non-fungal) cells O-glycosylate following a different mechanism, in that the initial step is the covalent attachment of N-acetyl-galactosamine to seryl or threonyl amino acids, no lipid-coupled oligosaccharide donor is involved in this first reaction, the initial step occurs in the Golgi, the structures of carbohydrates are different, etc.

In a preferred embodiment of the invention, the modified fungal cells carry genetic modification(s) in at least one gene whose

expression product is involved in the attachment of a mannosyl residue to the hydroxyl group of seryl or threonyl amino acids.

In a more preferred embodiment of the invention, the modified fungal cells carry genetic modification(s) in at least one gene whose expression product is involved in the transfer of a mannosyl residue from the Dol-P-Man precursor to the hydroxyl group of seryl or threonyl amino acids.

Still more preferably, one of these genes is the gene encoding the Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase [DPM2 - also designated PMT1] whose sequence is represented in Figure 4, or any homologous gene encoding the same activity as defined below.

In addition to modification(s) in one gene involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids, fungal cells of the invention may also carry modification(s) in the genes involved in subsequent additions of mannosyl residues leading to di- or oligosaccharides, or in the synthesis of the mannosyl residues donnor (Dol-P-Man).

Specific examples of such fungal cells are disclosed in the examples.

Another object of the invention resides in a fungal cell as disclosed above in which an exogenous DNA sequence has been introduced.

In the meaning of the present invention, the term exogenous DNA sequence includes any DNA sequence comprising one or more genes encoding a desired protein to be expressed and/or secreted in said cell. Such a DNA sequence may be a complementary DNA sequence (cDNA), an artificial DNA sequence, a genomic DNA sequence, a hybrid DNA sequence or a synthetic or semi-synthetic DNA sequence, included in an expression cassette enabling synthesis in the fungal cells of said proteins. The expression cassette preferably comprises a transcription and translation

initiation region joined to the 5' end of the sequence encoding said desired protein(s) so as to direct, and optionally regulate, the transcription and translation of said sequence. The choice of these regions may vary according to the fungal cell used. Generally, these sequences are chosen from promoters and/or terminators derived from fungal cell genes, and, when expression in yeast hosts is sought, from yeast genes. Of special interest are certain promoter and/or terminator regions derived from glycolytic genes of fungal cells such as, for yeasts, the genes encoding phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GDP), enolases (ENO) or alcohol dehydrogenases (ADH), and for filamentous fungi, the genes encoding triose phosphate isomerase (tpi). The promoter and/or terminator regions may also derive from other strongly expressed genes such as, for yeasts, the lactase gene (LAC4), the acid phosphatase gene (PHO5), the alcohol oxidase gene (AOX) or the methanol oxidase gene (MOX), and, for filamentous fungi, the cellobiohydrolase gene (CBHI), the alcohol dehydrogenase gene (alcA, alcC), the glucoamylase gene (GAM) or the acetamidase gene (amds), and the like. These transcription and translation initiation regions may be further modified, e.g. by *in vitro* mutagenesis, by introduction of additional control elements or synthetic sequences, or by deletions. For example, transcription-regulating elements, such as the so-called UAS, originating from another promoter may be used to construct hybrid promoters which enable the growth phase of the fungal cell culture to be separated from the phase of expression of the desired protein(s) encoding sequence(s). A transcription and translation termination region functional in the intended fungal cell may also be positioned at the 3' end of the coding sequence. In addition, at the N-terminus of the protein sequence, a signal peptide (pre-sequence) may be introduced so as to direct the nascent protein to the secretory pathway of the fungal cell used. This pre-sequence may correspond to the natural pre-sequence of the protein if this protein is naturally secreted, or it may be of another origin, e.g. obtained from another gene, or even artificial.

Preferably, the exogenous DNA sequence is part of a vector, which may either replicate autonomously in the fungal cell used or integrate into its own DNA sequences (chromosome). Autonomously replicating vectors may contain autonomously replicating sequences derived from the chromosomal DNA of the fungal cell (ARS) or from naturally-occurring fungal cell plasmids such as pGK1-1 [de Louvencourt et al., J. Bacteriol. 154 (1982), 737], pKD1 (EP 241 435), 2μm plasmid (Broach, Cell 28 (1982), 203-204) and the like. Integrating vectors usually contain sequences homologous to regions of the fungal cell chromosome which, after being introduced into said cell, enable integration through *in vivo* recombination. In a specific embodiment of the invention, said homologous sequences correspond to the region of the chromosome to be modified in the fungal cell, enabling a one-step modification-integration mechanism. Integration may also occur through non-homologous recombination.

The exogenous DNA sequence can be introduced into the fungal cell by any technique known in the art, and, for example, by recombinant DNA techniques, genetic crossings, protoplast fusions, etc. Concerning recombinant DNA techniques, transformation, electroporation, or any other technique disclosed in the literature may be used. More specifically, when the fungal cell is a yeast cell, the transformation may be performed according to the methods of Ito et al., [J. Bacteriol. 153 (1983), 163], Durr ns et al. [Curr. Genet. 18 (1990), 7] or following the method disclosed in EP 361 991. Electroporation can be performed according to Karube et al. [FEBS Letters 82 (1985), 90].

The fungal cells of the present invention can be advantageously utilized as host cells for the production of recombinant products such as heterologous proteins having pharmaceutical and/or agro-foodstuff interest. The fungal cells of this invention are particularly advantageous since they enable the production and/or secretion of high quality products, and since their genetic modifications do not affect the mitotic or genetic stability of said products' expression vectors. The cells of this invention are more particularly suitable for the production of pro-

teins having human therapeutic uses and which are susceptible to O-glycosylation by the host cell.

Accordingly, a further object of this invention resides in a process for the production of recombinant products wherein a fungal cell as defined above is cultivated in conditions in which the exogenous DNA sequence is expressed and the product is recovered. In a preferred embodiment, said product is secreted into the culture medium. In another preferred embodiment, said product is susceptible to O-glycosylation by the host cell.

The following proteins are cited as examples of heterologous proteins which can be prepared with the fungal cells of the present invention: enzymes (such as superoxide dismutase, catalase, amylases, lipases, amidases, chymosine, etc., or any fragment or derivative thereof), blood derivatives (such as human serum-albumin, alpha- or beta-globin, factor VIII, factor IX, van Willebrand factor, fibronectin, alpha-1 antitrypsin, etc., or any fragment or derivative thereof), insulin and its variants, lymphokines [such as interleukins, interferons, colony stimulating factors (G-CSF, GM-CSF, M-CSF...), TNF, TRF, etc., or any fragment or derivative thereof], growth factors (such as growth hormone, erythropoietin, FGF, EGF, PDGF, TGF, etc., or any fragment or derivative thereof), apolipoproteins, antigenic polypeptides for the preparation of vaccines (hepatitis, cytomegalovirus, Eppstein-Barr, herpes, etc.), or any fusion polypeptide such as, for example, fusions comprising an active moiety linked to a stabilizing moiety.

Another object of the invention resides in a DNA fragment encoding an enzyme involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids of proteins. Applicant has provided DNA fragments encoding such enzymes for the first time. More preferably, said DNA fragment comprises the Dol-P-Man:Protein (Ser/Thr) mannosyltransferase gene whose sequence is represented in Figure 4, any homologous gene, derivative or fragment thereof.

In the meaning of the present invention, homologous gene means any other gene of any fungal cell encoding an enzyme having the required activity. Said other genes may be obtained, for example, by complementation of a mutant fungal cell deficient in said activity with DNA prepared from a fungal cell capable of said activity, selection of the transformants having recovered the activity, and isolating their inserted DNA sequence. These other genes may also be isolated from DNA libraries by hybridization with probe(s) (including PCR primers) comprising all or part of the sequence presented in Figure 4. In this respect, it is also an object of this invention to use the DNA fragments provided, or any part thereof, as hybridization probe(s) or for the complementation of mutant phenotypes, for the obtention of homologous genes of fungal cells.

The term derivative means any other DNA fragment prepared by any genetic and/or chemical modification(s) of the genes mentioned above. Said genetic and/or chemical modification(s) may be any suppression, substitution, deletion or addition of one or more bases or of a region of said genes, leading either to an increased enzyme activity or to the same activity level, or to a decreased or null enzyme activity upon transformation in a fungal host cell.

LEGEND OF THE FIGURES

Figure 1: Restriction map of plasmids pDM3, pMT4 and pMT1.
Figure 2: Subcloning of plasmid pDM3.
Figure 3: Strategy of sequencing of the PMT1 gene.
Figure 4a: Nucleotide sequence of the PMT1 gene (SEQ ID N°1).
Figure 4b: Amino acid sequence of the PMT1 gene (SEQ ID N°2).
Figure 5: Construction and restriction map of pMT1.1/URA3.
Figure 6: O-glycosylation activity of *S. cerevisiae* WT (panel A) and MT (panel B).
Figure 7: Partial nucleotide sequence of the *K. lactis* PMT1 gene (SEQ ID No. 3).
Figure 8: Nucleotide (Panel A) and predicted amino acid (Panel B) sequence comparison between the *S. cerevisiae* PMT1 gene (upper sequences) and the *K. lactis* homolog (lower sequences) isolated by PCR amplification of *K. lactis* genomic DNA. Dots represent sequence identity,

question marks indicate sequence ambiguity. The nucleotide sequence complementary to the primer Sq3910 is underlined.

EXAMPLES

Example 1: Isolation of a highly purified mannosyltransferase from *S. cerevisiae* and generation of peptides

The mannosyltransferase activity was solubilized from total yeast membranes and purified on hydroxylapatite according to Strahl-Bolsinger and Tanner (Eur. J. Biochem. 196 (1991), 185). The protein then had to be further enriched by $(NH_4)_2SO_4$ precipitation before additional purification was performed via affinity chromatography. The eluted material was then separated on SDS/PAGE. The resulting 92 kDa band was cut out of the gel. Trypsin digestion (in the gel) yielded several non-overlapping peptides, enabling designing of probes.

E.1.1. $(NH_4)_2SO_4$ precipitation

100 ml of fractions of the hydroxylapatite column containing mannosyltransferase activity was mixed with $(NH_4)_2SO_4$ up to a final concentration of 30% (w/v) and stirred gently for 1 h in an ice/salt bath. The mixture was centrifuged for 30' (8000 x g). The resulting pellet was resuspended in 8 ml AB-buffer (10 mM Tris/HCl, pH 7.5, 15% glycerol (vol%), 0.1% lubrol (vol%), 150 mM NaCl) and dialyzed for 1 h against the same buffer. Storage: -20°C.

E.1.2. Affinity chromatography

E.1.2.1. Preparation of the affinity chromatography column

0.5 g freeze-dried powder of Protein A-Sepharose Cl 4B was swollen in 10 ml 100 mM NaPi, pH 7.0, for 15' and washed on a sintered glass filter (G3) with 200 ml of the same buffer. Protein A-Sepharose Cl 4B was equilibrated in 100 mM NaPi, pH 7.0. About 3 to 6 ml anti-mannosyltransferase serum was dialyzed for 2 h against 1 l NaPi (100 mM), pH 7.0. The dialyzed serum was incubated with the column material for 16 h at 4°C. The serum was

removed using a sintered glass filter (G3). The column material was washed twice with 10 ml 100 mM NaPi, pH 8.5, and resuspended in 50 ml of the same buffer.

For covalent coupling 0.75 mg/ml dimethylsuberimidate was added. The pH was adjusted to pH 8.5 by adding 5-6 drops of 1 M NaOH. The material was incubated for 1 h at RT. For a second time, dimethylsuberimidate was added and the pH adjusted to pH 8.5 with 1 M NaOH. The column material was washed serially on a sintered glass filter (G3) with:

a) 50 ml 100 mM NaPi, pH 8.0
b) 25 ml 100 mM NaPi, pH 8.0, 3 M ammonium rhodanide
c) 100 ml 100 mM NaPi, pH 8.0

The material was washed and equilibrated in AB-buffer.

E.1.2.2. Purification of the 92 kDa protein

8 ml of the $(NH_4)_2SO_4$ precipitated and dialyzed protein (E.1.1.) was incubated with the affinity column material (E.1.2.1) for 16 h at 4°C with gentle shaking. A column (2 cm x 0.5 cm) was filled and washed with 15 ml AB-buffer. The column was eluted with 100 mM glycine/HCl pH 3.0, 0.05% lubrol (vol%), 15% glycerol (vol%). Fractions of 0.9 ml were collected and neutralized immediately with 1 M Tris (15 µl/0.9 ml fraction).

To detect the 92 kDa protein, 40 µl of each eluted fraction was analyzed by SDS/PAGE and Western-blot analysis as described (Strahl-Bolsinger and Tanner, 1991). The 92 kDa protein containing fractions (fraction 2-6) were pooled and concentrated to 100 µl via microconcentrators (Centricon/Amicon) by centrifugation at 5000 x g. 0.9 ml 98% EtOH were added and the protein precipitated for 16 h at -20°C. The precipitated protein was pelleted by centrifugation for 30' at 10,000 x g.

E.1.3. SDS-PAGE

The precipitated protein (E.1.2.2) was resuspended in 150 µl SDS-sample buffer (0.07 M Na_2CO_3, 0.07% ß-EtSH, 2% SDS, 12% Saccharose, 0.07% bromphenolblue). SDS-gel electrophoresis according to Lämmli and Favre [J. Mol. Biol. 80 (1973), 575] was carried out at 50-70 V using the BIORAD-Mini-Protean cell. Protein standards: HMW-Standards/Gibco BRL.

Protein was detected by staining with 0.05% Coomassie R250 (w/v), 25% isopropanol (vol%), 10% acetic acid (vol%) and destaining in 7.5% acetic acid (vol%).

E.1.4 Trypsin digestion and designing of oligonucleotides

After SDS-PAGE (E.1.3.) the 92 kDa protein band was cut out (about 10 µg of protein). The gel fragment was cut into small pieces and shaken three times for 30' in 5 ml 50% methanol/10% acetic acid and one time for 30' in 5 ml 50% methanol. The gel was lyophylized for 3 h. Trypsin digestion was carried out in 0.3 ml 0.2 M ammoniumhydrogen carbonate/2µg trypsin for 16 h at 37°C. Supernatant was removed. Elution of the peptides was done three times for 1 h at 37°C in 0.2 ml 0.2 M ammoniumhydrogen carbonate and one time for 1 h at 37°C in 0.2 ml 0.2 M ammonium-hydrogen carbonate/30% acetonitrile. The eluted material was pooled, lyophylized and resolved in 0.2 ml 1M guanidinium hydrochloride/50 mM Tris/HCl, pH 7.5. Peptides were separated using a reverse phase RP18 column equilibrated in 0.13% TFA. Peptides were eluted by acetonitrile (0-70%). Up to 40 different peptide peaks could be detected. Five of the main peaks were sequenced via automated sequence analysis according to Edman (G. Allen in: Sequencing of proteins and peptides, Laboratory Techniques in Biochem. and Mol. Biol. 9 ed.: Burdon, R.H. & Knippenberg, P.H.; Elsevier (1989)). Among the sequences thereby obtained, three were suitable for designing oligonucleotides, which are presented in Table 1, below.

Table 1

Peak	Peptide Sequence
15	I S Y K P A S F I S K
23	E V S P Y G Y S G F D G D A
34	N L V E P H V Y E S

On the basis of these sequences, oligonucleotides A-C were chemically synthesized, using the codon usage of *S. cerevisiae* (Guthrie and Abelson in: The molecular biology of the yeast Saccharomyces; eds: J.N. Strathern, E.W. Jones, J.R. Broach (1982)). Oligonucleotides A-C have the following characteristics:

- Oligonucleotide A:
  peak: 23
  amino acid sequence: G F D G D A
  Oligodeoxynucleotide: 5'-G$^{T}/_{C}$GTCACCGTCGAANCC-3'
  8-fold degenerated, coding strand, 17 nucleotides

- Oligonucleotide B:
  Peak: 34
  Amino acid sequence: E P H V Y E
  DNA sequence: 5'-$^{C}/_{T}$TCGTAGAC$^{G}/_{A}$TG$^{A}/_{T}$GG$^{T}/_{C}$TC-3'
  16-fold degenerated, coding strand, 18 nucleotides

- Oligonucleotide C:
  Peak: 15
  Amino acid sequence: I S Y K P A S F I S K
  DNA sequence: 5'-ATTTC$^{T}/_{A}$TA$^{T}/_{C}$AA$^{A}/_{G}$CC$^{A}/_{T}$GCTTC$^{T}/_{A}$
  TT$^{T}/_{A}$AAA-3'
  128-fold degenerated, coding strand, 33 nucleotides

Example 2: Screening of a plasmid library of yeast genomic DNA

The chemically synthesized oligodeoxynucleotides A-C (E.1.4.) were used to screen the plasmid library of yeast genomic DNA pCS19 (Sengstag and Hinnen, Nucl. Acids Res. 15 (1987), 233). This library was prepared by partial digestion of yeast genomic DNA with Sau3A, and cloning into the BclI restriction site of the vector pCS19.

E.2.1. Labeling of Oligodeoxynucleotides

The oligonucleotides A-C were labeled by kinase reaction, carried out according to Maniatis et al (T. Maniatis, J. Sambrook, E.F. Fritsch (1989), Molecular cloning: A Laboratory manual, C.S.H. Press). 40 pmol Oligodeoxynucleotide were labeled using 50 μCi [γ-^{32}P]-ATP. Free radioactive nucleotides were removed using "NUC Trap Push columns" (Stratagene) according to the instruction manual of the producer.

E.2.2. Screening of the library

The DNA-library (4992 different single colonies) was transferred from microtiterplates to nitrocellulose. Colony hybridization was performed according to Grunstein and Hogness (PNAS 72 (1975), 3961) in the following conditions:

- Prehybridization: Filters were incubated at 44°C in 200 ml 5 x Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA, for at least 4 h (5 x Denhardt's: 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA; 6 x NET: 0.9 M NaCl, 90 mM Tris-HCl, pH 8.3, 6 mM EDTA, pH 8.0).

- Hybridization: Filters were incubated at 44°C in 100 ml 5 x Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA, labeled oligodeoxynucleotides A and B (40 pmol each). Hybridization was performed for 16 h.

- Washing conditions: Filters were washed three times in 50 ml 6 x SSC, 0.1% SDS (w/v) at 0°C for 15'.

To detect positive colonies, the filters were exposed to X-ray films for 16 h, -70°C. Under these conditions, 12 positively reacting clones could be identified.

Example 3: Southern Analysis of the 12 positive clones

The 12 positive clones were analyzed in Southern blots using three different oligodeoxynucleotides. This analysis led to the identification of one positive clone reacting with all three oligonucleotides. This clone was called pDM3.

The 12 positive clones were grown in 5 ml LB medium supplemented with ampicillin and their DNA was isolated according to the method of Birnbaum and Doly (Nucl. Acid. Res. 7, (1979), 1513).

1/10 of each isolated plasmid DNA (plasmids: pDM1-pDM12) was digested with the restriction enzymes EcoRI-XhoI (5U each), 1 x "one for all" buffer (Pharmacia) in a total volume of 20 µl for

1 h at 37°C. DNA fragments were separated on a 1% agarose gel and blotted to nitrocellulose according to Maniatis et al. (loc. cit.). Southern analysis was performed using oligo A and B using the same conditions as described for the library screen. The hybridization temperature for oligo A was 48°C, for oligo B 42°C. Clones 1, 2, 3, 5, 6, 7 and 11 reacted positively with both oligodeoxynucleotides. These seven clones were further analyzed by Southern blot analyses. Three identical blots were therefore prepared, in which the DNA of clones 1, 2, 3, 5, 6, 7 and 11 was digested with EcoRI-XhoI and blotted to nitrocellulose as described. Blots 1, 2 and 3 were prehybridized in 20 ml 5 x Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA at 50°C for 4 h. Each blot was then hybridized in 10 ml 5 x Denhardt's, 6 x NET, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA, 40 pmol labeled oligonucleotides for 16 h. The hybridization temperature is indicated in Table 2, below. Washing was performed for 10' at each temperature in 50 ml 2 x SSC, 0.1% SDS (w/v).

Table 2

Blot	Hybridization with oligo	Hybridization temperature	Washing conditions	Positive reacting clones
Blot 1	A	25	2x10'/25°C 1x10'/35°C	1,2,3,5,6,7,11
Blot 2	B	25	2x10'/25°C 1x10'/35°C	3, 5
Blot 3	C	45	2x10'/45°C 1x10'/55°C	3

Clone 3 was the only clone reacting with oligo A, B and C. The clone was called pDM3 and further analyzed.

Example 4: Analysis of pDM3

E.4.1. Methods

E.4.1.1 Digestion with restriction endonucleases

Analytic digestion with endonucleases was performed in 1 x "one for all" buffer (Pharmacia), 0.2 - 0.5 µg of DNA, 1-5 U restriction enzyme in a total volume of 20 µl for 1 h at 37°C.

Preparative digestion was performed in a total volume of 40-80 µl with 1-10 µg of DNA, 5-20 µl restriction enzyme, 1 x "one for all" buffer for 2 h at 37°C.

E.4.1.2. DNA-gelelectrophoresis

Separation of DNA fragments was performed according to Maniatis et al. (loc. cit.).

E.4.1.3. Isolation of DNA fragments

After separation, DNA fragments were isolated using the "Geneclean kit" (Stratagene) according to the instruction manual of the producer.

E.4.1.4. Treatment with alkaline phosphatase

DNA fragments were dephosphorylated with alkaline phosphatase according to Maniatis et al. (loc. cit.).

E.4.1.5. Ligation

DNA fragments were ligated in 1 x T4-ligation buffer (50 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP) with 1 U T4-DNA ligase (total volume 10-15 µl). Molar DNA ratio of vector: insert was 1:4 or 1:8. The absolute amount of DNA was 20-50 ng. Incubation time: 16 h at 14°C or 5 h at 25°C.

E.4.1.6. Transformation of *E. coli*

Competent *E. coli* DH5α cells were prepared according to Hanahan (J. Mol. Biol. 166 (1983), 557). Transformation was carried out as described by Maniatis et al. (loc. cit.).

E.4.1.7. Preparation of DNA

Plasmid DNA was prepared according to Birnbaum and Doly (loc. cit.).

E.4.1.8. Southern blot analysis

Southern blot analysis was performed using the same condition as described in E.3.

E.4.1.9. DNA sequence analysis

DNA sequencing was done according to the method of Sanger et al. (PNAS 74 (1977), 5463). Only plasmid DNA was sequenced. T7-DNA polymerase sequencing kit (Pharmacia) was used; the radioactive nucleotide was [α-^{35}S]-dATP (spec. act. 600 Ci/mmol).

E.4.2. Identification of the ORF

This example discloses a restriction analysis of pDM3, the identification of different DNA fragments recognized by oligonucleotides A, B or C and their subcloning. Sequencing of these subclones enabled identification of an ORF.

E.4.2.1. Subcloning of pDM3 DNA fragments hybridizing with oligo A, B or C.

pDM3 DNA was digested with EcoRI, XhoI and EcoRI-XhoI. Southern blot analysis was performed using oligo A, B or C as a target.

Oligo A recognizes a 3.0 kb EcoRI fragment, oligo B and C recognize a 1.1 kb EcoRI-XhoI fragment. The 3.0 kb EcoRI fragment was subcloned into pUC19 (linearized with EcoRI and dephosphorylated). The 1.1 kb EcoRI-XhoI fragment was subcloned into pUC18 (linearized with EcoRI-SalI, and dephosphorylated). Right subclones were identified by restriction analyses and Southern blot analysis using oligo A or B/C, respectively.

The 3.0 kb EcoRI subclone was called pMT4, the 1.1 kb EcoRI-XhoI subclone was called pMT1. Further restriction analysis of pMT4 and pMT1 was performed using a number of different restriction

endonucleases (for example: PstI, HindIII and BglII). Southern blot analysis using oligo A or B/C was carried out to define the exact region of a possible ORF.

Restriction maps of pDM3, pMT4 and pMT1 are shown on Figure 1.

E.4.2.2. Sequence analysis

From both ends, the DNA inserts of plasmids pMT4 and pMT1 were sequenced using the universal and reverse primers, priming next to the polylinker of pUC19/pUC18. Also oligos A, B and C were used as sequencing primers. The sequencing data resulted in an ORF of about 400 bp on both sides of the insert of pMT1. Also pMT4 showed an ORF of about 200 bp when sequenced with the reverse primer. Using these sequencing data, an amino acid sequence could be deduced. This AA-sequence showed peptide sequences known from the peptide analysis of the 92 kDa protein (peptides corresponding to peaks 15, 23, 34 were found). According to these data, the 5'/3' orientation of the gene could be predicted.

Several other subclones were constructed and sequenced using the universal and reverse primers of pUC18/19 (Figure 2).

The following oligodeoxynucleotides were also used for sequencing:

Oligo	Sequence (5'-3')	vector sequenced
Oligo 6	CCAACCAGACAACCACTGGG 2713	pMT1
Oligo 7	GACAGGCCACTAACAGCTTC 697	pMT4
Oligo 8	GATGTTGTATGCTGGTGTG 840	pMT4
Oligo 9	CATTGAGCGAGTTGGCAGGG 1178	pMT4
Oligo 4	GAACCTCATGTTTATGAA 2189	pMT1

These oligodeoxynucleotides represent parts of the newly sequenced DNA fragments.

For sequencing the 5' region of the gene, exoIII/mung bean deletions of the vector pMT4 were made. pMT4 was linearized using SphI (3' overlap). The plasmid was then cut using BamHI (5' overlap).

Exonuclease III deletion was performed according to Roberts and Lauer (Meth. Enzymol. 68 (1979), 473), Henikoff (Meth. Enzymol. 155 (1987), 156).

Overlapping ends were removed by mung bean nuclease. The resulting plasmids were analyzed by restriction analysis using HindIII and EcoRI.

Sequence analysis of the clones was carried out using the reverse primer of pUC19. The sequence strategy is shown in Figure 3. Sequence data are given in Figure 4.

Example 5: Northern blot analysis: Identification of mRNA encoding Mannosyltransferase.

E.5.1. Methods

E.5.1.1. Isolation of RNA

Total RNA was isolated from yeast strain SEY2101 (Mat a, ade2-1, leu2-3, 112, ura3-52 (Emr et al. PNAS 80 (1983), 7080) according to Domdey et al. (Cell 39 (1984), 611).

E.5.1.2. Northern blot

Total RNA was separated using a formaldehyde agarose gel and blotted to nitrocellulose as described by Maniatis et al. (loc. cit.).

E.5.1.3. The DNA-target

The 1.1 kb insert of pMT1 was isolated by EcoRI-PstI digestion. The fragment was purified using the "Gene-clean kit" (Stratagene).

200 ng of the DNA fragment were labeled with [α-^{32}P]-dCTP (50 μCi) using the "megaprime" labeling kit (Amersham) according to the instruction manual of the producer.

E.5.2. Results

The nitrocellulose filter was prehybridized for 2 h at 42 C in 20 ml 5 x Denhardt's, 2 x SSC, 0.1% SDS (w/v), 50% formamide (v/v), 0.1 mg/ml salmon sperm DNA. Hybridization was performed at 42°C for 16 h in 10 ml 1 x Denhardt's, 2 x SSC, 0.1% SDS (w/v), 50% formamide (v/v), 0.1 mg/ml salmon sperm DNA, 200 μg [α-^{32}P]-dCTP labeled 1.1 kb EcoRI-PstI fragment of pMT1. Washing was done twice at RT and two times at 50°C in 50 ml 0.1 x SSC, 0.1% SDS (w/v). Hybridization of the target was detected by exposure to X-ray film (-70°C, 16 h). A single mRNA with the size of 3 kb was detected.

This procedure may be easily repeated by the person skilled in the art with other probes derived from the sequence of Figure 4 and with RNA from other sources (other fungal cells).

Example 6: Preparation of an *S. cerevisiae* cell deficient in O-glycosylation activity.

An *S. cerevisiae* cell deficient in O-glycosylation activity was prepared by gene disruption, by insertion of the URA3 gene into the HindIII restriction site of the identified ORF, at bp 1595 of the coding sequence.

E.6.1. Construction of the plasmid used for the gene disruption

The 1.1 kb insert of pMT1 was isolated as EcoRI-PstI fragment and subcloned into a pUC18 vector (EcoRI/PstI linearized, dephosphorylated, without HindIII restriction site in the polylinker). The resulting vector was called pMT1.1.

pMT1.1 was linearized with HindIII and dephosphorylated. The 1.1 kb HindIII fragment of YEp24 (Julius et al., Cell 37 (1984), 1075) containing the URA3 gene of *S. cerevisiae* was isolated and subcloned into the HindIII linearized, dephosphorylated vector pMT1.1. Clones were identified by restriction analyses and called pMT1.1/URA3 (Figure 5).

pMT1.1/URA3 has 0.24 kb PMT1 coding sequence flanking one side of the URA3 gene and 0.86 kb PMT1 coding sequence flanking the other. CsCl-DNA of pMT1.1/URA3 was prepared according to Maniatis et al. (loc. cit.).

E.6.2. Transformation of yeast

40 µg of pMT1.1/URA3 CsCl-DNA was digested with SphI/EcoRI. To check that the digestion was complete, part of the digested DNA was analyzed on a DNA agarose gel. The digest was then phenolized and the DNA precipitated with 98% EtOH (Maniatis et al., loc. cit.). DNA was resolved in 10 µl TE, pH 8.0.

S. cerevisiae strains SEY2101/2102 (Mat a/α, ura3-52, leu2-3, 112 (Emr et al., loc. cit.) and SEY2101 (Mat a, ura3-52, leu2-3, 112, ade2-1) were transformed with 5 µl of the EcoRI/SphI digested vector pMT1.1/URA3 according to the method of Ito et al. (J. Bacteriol. 153 (1983), 163).

SEY2101/2102 transformants were selected on minimal media + Leu; SEY2101 transformants were selected on minimal media + Leu, + Ade.

After 3-4 days at 30°C, transformants could be picked and plated on the same media for a second time.

E.6.3. Genomic Southern blot of the transformants

Genomic DNA of three haploid transformants and wild-type cells was isolated as described by Hoffmann and Winston (Gene 57 (1987), 267). 1 µg of the genomic DNA was digested with XhoI/EcoRI, separated on an agarose gel and blotted to nitrocellulose as described by Maniatis et al. (loc. cit.).

The blot was prehybridized in 20 ml 5 x Denhardt's, 2 x SSC, 0.1% SDS (w/v), 0.1 mg/ml salmon sperm DNA, 50% formamide (w/v) for 4 h at 42°C.

Hybridization was permitted in 10 ml of the same solution adding 200 ng [α-^{32}P]-dCTP labeled 1.1 kb EcoRI/PstI fragment of pMT1.1 (see: E.5.1.3) for 16 h at 42°C. Washing was done two times at RT in 50 ml 2 x SSC, 0.1% SDS (w/v) and two times at 68°C in 50 ml 1 x SSC, 0.1% SDS (w/v). Signal detection by X-ray films. Wild-type cells showed a single signal at 1.1 kb, reflecting the EcoRI/XhoI fragment without URA3 insertion. In the disrupted strains this signal was missing. Instead of this, a new 2.2 kb fragment was recognized by the 1.1 kb target, representing the 1.1 kb EcoRI/XhoI fragment bearing the 1.1 kb URA3 insertion.

Example 7: Characterization of the mutant

E.7.1. Growth

SEY2101 wild-type cells were grown either on YPD (10 g/l yeast extract; 10 g/l peptone; 20 g/l dextrose) or on minimal media + Ade, + Leu, + Ura. SEY2101 PMT1::URA3 mutant cells were grown either on YPD or on minimal media + Ade, + Leu. Cells were grown at 30°C in a waterbath shaker. OD578 was measured every 30' after sonifying cells. Wild-type and mutant cells show nearly identical growth on both media although, in some cases, mutant cells may stick together. Nevertheless, such cells can easily be separated by sonifying (30", sonify water bath). The growth characteristics of these cells are listed below:

- Generation time:
  - WT : 99'
  - MT : 93'
- Cell number:
  - WT : 1 OD = 1.9 x 10^7
  - MT : 1 OD = 1.9 x 10^7
- Doubling rate:
  - WT: 0.61/h
  - MT: 0.65/h

In a logarithmically growing culture, 54.7% of wild-type cells and 56% of mutant cells show buds. After growing for 24 h on YPD wild-type cells reached an OD578 of 11.4 and mutant cells of 12.3.

E.7.2. *In vitro* mannosyltransferase activity and Western blot

E.7.2.1. Preparation of crude membranes

SEY2101 was grown in 100 ml minimal media + Ade + Leu + Ura to OD578 = 0.5. SEY2101 PMT1::URA3 was grown in 100 ml minimal media + Ade, + Leu to OD578 = 0.5.

Two preparations of each strain were carried out. Work was performed on ice; all buffers were at 4°C. 40 OD of cells were pelleted and washed in 25 ml TMA (50 mM Tris/HCl, pH 7.5, 7.5 mM $MgCl_2$). Cells were resuspended in 100 µl TMA and transferred to a violax tube. 0.3 g of glass beads were added and cells broken on a vortex four times for 30" (cooling on ice between breaking intervals). The extract is removed from glass beads using a pasteur pipette. Glass beads are washed three times with 250 µl TMA. All washing solutions are pooled in an Eppendorf cup. The solution is centrifuged for 15" (10,000 x g). Supernatant is removed and the pellet resuspended in 40 µl TMA (1 OD = 1 µl)

E.7.2.2. Mannosyltransferase assay (*in vitro*)

1 and 5 µl of the crude membranes (E.7.2.1) were tested for enzyme activity as described by Strahl-Bolsinger and Tanner (loc. cit.). Two parallel samples from wild-type and mutant cells were measured. Mean values of these two independent measurements are shown.

	µl membranes	cpm/incubation[1)]
WT	1	2786
WT	5	10289
MT	1	563
MT	5	1135

1) Control values (without peptide) were 506 and 1031, respectively.

In contrast to wild-type cells, mutant cells show no *in vitro* mannosyltransferase activity.

E.7.2.3. Western blot analysis

Membranes (1 µl of E.7.2.1) were incubated in 20 µl SDS sample buffer for 1 h at RT. Then SDS/Page and western blot were performed as described by Strahl-Bolsinger and Tanner (loc. cit.). For antibody detection the Peroxidase ECL kit (Amersham) was used according to the instruction manual of the producer. Antibodies against the 92 kDa protein react specifically with a 92 kDa protein of wild-type membranes. In mutant membranes this 92 kDa signal is missing.

E.7.3. *In vivo* O-glycosylation

To investigate *in vivo* glycosylation, wild-type and mutant cells were grown in the presence of [^{3}H]-mannose. Then a crude cell wall plus membrane fraction was isolated and O-glycosylated material released by ß-elimination.

E.7.3.1 Treatment with [^{3}H]-mannose

Wild-type and mutant cells were grown over night in minimal media containing sucrose as only C-source. 7.5 OD of the culture (OD578 = 1-2) were pelleted and washed with 5 ml H_2O (prewarmed 30°C). The cells were grown in 5 ml YP/0.5% sucrose/250 µCi [^{3}H]-mannose in a waterbath shaker for 2 h at 30°C.

E.7.3.2. Isolation of crude cell wall and membrane fraction

5 OD of the [^{3}H]-mannose treated cells were centrifuged and washed three times with 1 ml TMA. Cells were resuspended in 200 µl TMA and broken with glass beads as described in E.7.2.1 (10

µl sample was used for counting radioactivity corresponding to total incorporation). The extract was then centrifuged for 15' (10,000 x g) and the supernatant was removed (100 µl sample was used for counting radioactivity corresponding to soluble material).

E.7.3.3. ß-elimination

The pellet was resuspended in 1 ml 0.1 N NaOH (a 10 µl sample was used for counting radioactivity corresponding to material before ß-elimination). Incubation was maintained for 24 h at 30°C.

E.7.3.4. Analysis of ß-eliminated material

ß-eliminated material was desalted via a Dowex 50WS8/H^+ column (0.5cm x 6cm). The column was saturated with 0.5M mannose and equilibrated in H_2O. The ß-elimination sample was loaded onto the column and washed through with 1.5 µl H_2O. The flow through was collected (a 100 µl sample was used for counting radioactivity corresponding to ß-eliminated material) and concentrated to 10 µl in the speed-vac. Thin-layer chromatography on Silicagel 60 (Merck) in acetone:butanol:H_2O 70:15:15 was performed. Standards: mannose, sucrose, stachyose, raffinose. The chromatographic run was repeated once. Sugars were detected with 0.5 g $KMnO_4$ in 100 ml 1N NaOH. Radioactivity was detected by a thin-layer scanner (Berthold) (see Figure 6).

Cell/ cpm	Total incorporation	Soluble material	Material before ß-elimination	ß-eliminated radioactivity
WT	1.46×10^7	2.23×10^6	1.2×10^7	1.2×10^6
MT	1.12×10^7	1.84×10^6	0.79×10^7	4.0×10^5

Mutant cells show reduced glycosylation in comparison to wild-type cells. The O-glycosylation in mutant cells is about 40-50% lower than in wild-type cells.

Example 8: Cloning of the PMT1 homolog of *Kluyveromyces lactis*.

The yeast *S. cerevisiae* used to be the system of choice when the production of a heterologous protein in a fungal host cell was desired. However, recent years have shown that the productivity of bakers yeast is often limited, especially when the secretion of the product to the culture medium is required, The use of fungal systems other than *S. cerevisiae* is therefore preferable in a number of cases [c.f. Romanos et al., Yeast 8 (1992) 423-488; Fleer, Curr. Opinion Biotechnol. 3 (1992) 486-496]. One of the alternative yeast hosts is represented by the genus Kluyveromyces for which superior secretion yields have been found with respect to several proteins of commercial interest [e.g. Van den Berg et al., Bio/Technology 8 (1990) 135-139]. The following example demonstrates that the present invention is not limited to bakers yeast, since the sequence of the PMT1 gene isolated from *S. cerevisiae* can be advantageously used for the identification of genes encoding similar enzymatic activities in other fungal species. Furthermore, the sequence information revealed in the present invention may also be used for the identification of related mannosyltransferase encoding genes in *S. cerevisiae*.

E.8.1. Design of degenerate PCR primers for the amplification of PMT1 related genes

The region of the PMT1 nucleotide sequence corresponding to the central, hydrophilic region of the mannosyltransferase protein was chosen to design PCR primers [Polymerase-catalyzed Chain Reaction, Saiki et al., Science 230(1985) 1350-1354; Mullis & Faloona, Meth. Enzymol. 155 (1987) 335-350] for the amplification of homologous genes. Amplification requires hybridization, also termed annealing, of these synthetic oligonucleotides with its target DNA. The specificity with which individual regions of genomic DNA are amplified depends on the conditions of the PCR reaction and the degree of homology between the primers and the nucleotide sequence to be amplified. Subsequent to the annealing step, the primers are extended using a thermostable DNA polymerase. Once the complementary strand has been polymerized,

the two strands are separated by heat denaturation and a new cycle of annealing and polymerization may begin.

Four examples of oligonucleotides suitable as primers for PCR amplification of PMT1 homologs are presented in Table 2 below.

Table 2

Primer Designation	Nucleotide Sequence
Sq3908	5'ATGGAYGCNAAYAAYGAYTGG-3'
Sq3909	5'-GAYGCNAAYGAYGAYTGGGT-3'
Sq3910	5'-TCYTGYTGYTCRAANCCCCA-3'
Sq3911	5'-CTRTTRTTYTCNCCCCARTA-3'

The design of these "degenerate" oligonucleotides takes into account that several codons may contain the information for the incorporation of the same amino acid into a nascent polypeptide chain, most often varying in the third ("wobble") position of a triplet. Each primer therefore represents a mixture of oligonucleotides where Y signifies C or T, R signifies A or G, and N signifies A, C, G or T.

E.8.2. PCR amplification of *K. lactis* genomic DNA

Genomic DNA of *K lactis* strain CBS2359 was prepared as described by Sherman et al. ["Methods in Yeast Genetics", Cold Spring Harbor Laboratory Press (1986) p 127]. 10ng of genomic DNA were used in a standard PCR reaction [Sambrock et al., "Molecular Cloning - A Laboratory Manual", second edition, Cold Spring Harbor Laboratory Press (1989)] in the presence of 1 μg of each of the primers and 5% deionized formamide. The amplification was performed using a "DNA Thermal Cycler" (Perkin Elmer Cetus) and "AmpliTag DNA Polymerase" (Perkin Elmer Cetus, 5 units per reaction tube). The conditions for denaturation, annealing, and polymerization (30 cycles) were 91°C (1 min), 42°C (2 min), and 72°C (3 min), respectively, except for the first cycle where

denaturation was for 5 min. The results of the PCR amplifications using the primers disclosed above are presented in Table 3 below.

Table 3

Primer Combinations	Approx. size of amplified DNA DNA fragments (bp)	
	expected for PMT1 homolog	observed
Sq3908+Sq3910	400	400
Sq3908+Sq3911	600	600
Sq3909+Sq3910	170 300	170
Sq3909+Sq3911	400 800	400

These results show that it is not only possible to obtain fragments exhibiting the same size as that expected for the *K. lactis* homolog of the *S. cerevisiae* PMT1 gene but that, in addition, DNA fragments can be amplified with high specificity that most likely correspond to another gene coding for a closely related enzymatic activity.

E.8.3 Partial sequence characterization of the *K. lactis* PMT1 homolog

The 400 bp fragment amplified with the primer combination Sq3908+Sq3910 was subcloned into the vector pCRII (TA Cloning™, Invitrogen Corp.) following the indications of the supplier, and partially sequenced according to the method described in E.4.1.9. using the universal primer. The sequence obtained is presented in Figure 7. Sequence comparison between the *S. cerevisiae* PMT1 gene and the fragment isolated from *K. lactis* genomic DNA by PCR amplification reveals 75% and 80.5% identity on the nucleotide and amino acid level, respectively (Figure 8). The amplified 400 bp DNA fragment may be used to target a selectable marker gene to the *K. lactis* PMT1 chromosomal locus

in analogy to the experiment described under E.6. leading to a disrupted gene. This will yield a *K. lactis* strain with reduced Ser/Thr specific mannosyltransferase activity. In addition, the amplified fragment may be used as homologous hybridization probe for the cloning of the entire *K. lactis* PMT1 gene using standard procedures.

CLAIMS

1. Fungal cells carrying genetic modification(s) within their DNA sequences which cause them to have at least a reduced capacity of O-glycosylation.

2. Fungal cell according to claim 1 wherein said modification(s) comprise any suppression, substitution, deletion, addition, disruption and/or mutational insertion.

3. Fungal cell according to claim 2 wherein said modification(s) are stable during segregation and/or non-reverting and/or non-leaky.

4. Fungal cell according to any of claims 1 to 3 wherein said modification(s) are located in one or more coding regions of the DNA sequences of the cell.

5. Fungal cell according to any of claims 1 to 3 wherein said modification(s) are located in one or more regions responsible for or involved in the expression and/or transcriptional regulation of a gene.

6. Fungal cell according to claim 4 or 5 wherein said gene is a gene whose expression product is an enzyme of the O-glycosylation pathway.

7. Fungal cell according to claim 6 wherein the reduced capacity of O-glycosylation results from the production of inactive enzymes, from the production of enzymes having altered biological properties, from the absence of production of said enzymes, or from the production of said enzymes at low levels.

8. Fungal cell according to claim 6 wherein said gene is a gene whose expression product is involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids.

9. Fungal cell according to claim 8 wherein said gene is a gene whose expression product is involved in the transfer of mannosyl residues from the Dol-P-Man donor to the hydroxyl group of seryl or threonyl amino acids.

10. Fungal cell according to claim 9 wherein said gene is the gene encoding the Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase [PMT1] whose sequence is represented in Figure 4, or any homologous gene encoding the same activity.

11. Fungal cell according to any of claims 8 to 10, further comprising modification(s) in one or more genes involved in subsequent additions of mannosyl residues, or in the synthesis of the mannosyl residues donor (Dol-P-Man).

12. Fungal cell according to any of the preceding claims in which an exogenous DNA sequence has been introduced.

13. Fungal cell according to claim 12 wherein the exogenous DNA sequence comprises one or more genes encoding a desired protein to be expressed and/or secreted in said cell.

14. Fungal cell according to claim 13 wherein said DNA sequence is included in an expression cassette comprising a transcription and translation initiation region joined to the 5' end of said DNA sequence encoding the desired protein(s).

15. Fungal cell according to claim 14 wherein said transcription and translation initiation region is chosen from promoters derived from fungal cell genes.

16. Fungal cell according to claim 14 wherein said expression cassette further comprises a transcription and translation termination region at the 3' end of the DNA sequence encoding the desired protein(s).

17. Fungal cell according to claim 14 wherein said expression cassette further comprises a signal peptide (pre-sequenc) at the N-terminus of the desired protein sequence so as to direct the nascent protein to the secretory pathway of said fungal cell.

18. Fungal cell according to any of claims 12 to 17 wherein the exogenous DNA sequence is part of a vector which may either replicate autonomously in said fungal cell or integrate into its own DNA sequences (chromosome).

19. Fungal cell according to any of the preceding claims wherein it is chosen from filamentous fungi and yeasts.

20. Fungal cell according to claim 19 wherein the filamentous fungi is chosen from the group consisting of *Aspergillus, Trichoderma, Mucor, Neurospora, Fusarium,* and the like.

21. Fungal cell according to claim 19 wherein the yeast is chosen from the group consisting of *Kluyveromyces, Saccharomyces, Pichia, Hansenula, Candida, Schizosaccharomyces* and the like.

22. Fungal cell according to claim 21 wherein the yeast is chosen from the group consisting of *Kluyveromyces, Saccharomyces, Pichia, Hansenula,* and *Candida,* and, more preferably, from *Kluyveromyces* and *Saccharomyces*.

23. Process for the production of recombinant products wherein a fungal cell according to claims 12-22 is cultivated in conditions in which the exogenous DNA sequence is expressed, and the product is recovered.

24. Process according to claim 23 wherein said product is secreted into the culture medium.

25. Process according to claims 23 or 24 wherein said product is susceptible to O-glycosylation by the fungal cell.

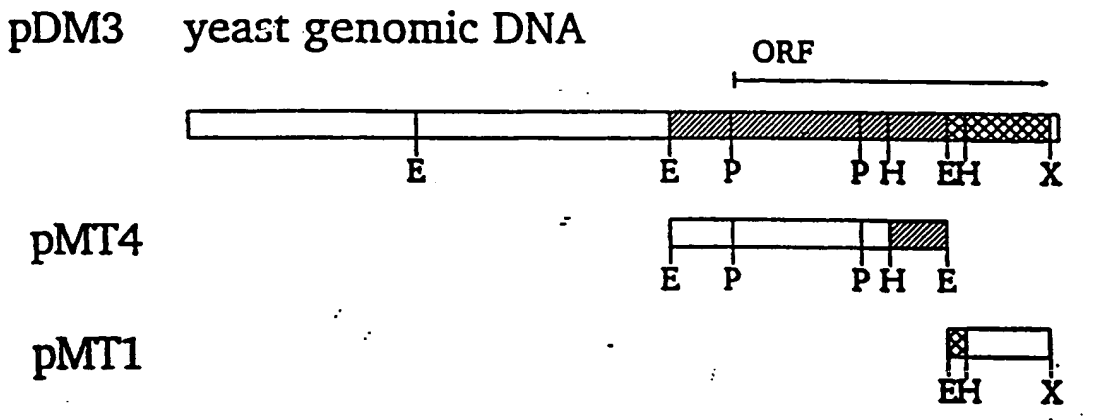
26. DNA fragment encoding an enzyme involved in the attachment of mannosyl residues to the hydroxyl group of seryl or threonyl amino acids of proteins.

27. DNA fragment according to claim 26 comprising the Dol-P-Man:Protein (Ser/Thr) Mannosyl Transferase gene whose sequence is represented in Figure 4, or any fragment or derivative thereof.

28. DNA fragment according to claim 26 comprising all or part of the sequence presented in Figure 7.

29. The use of a DNA fragment according to claim 26 or 27 or any part thereof as hybridization probe(s) or for the complementation of mutant phenotypes for the obtention of homologous genes of fungal cells.

30. DNA fragments Sq3908, Sq3909, Sq3910 and Sq3911.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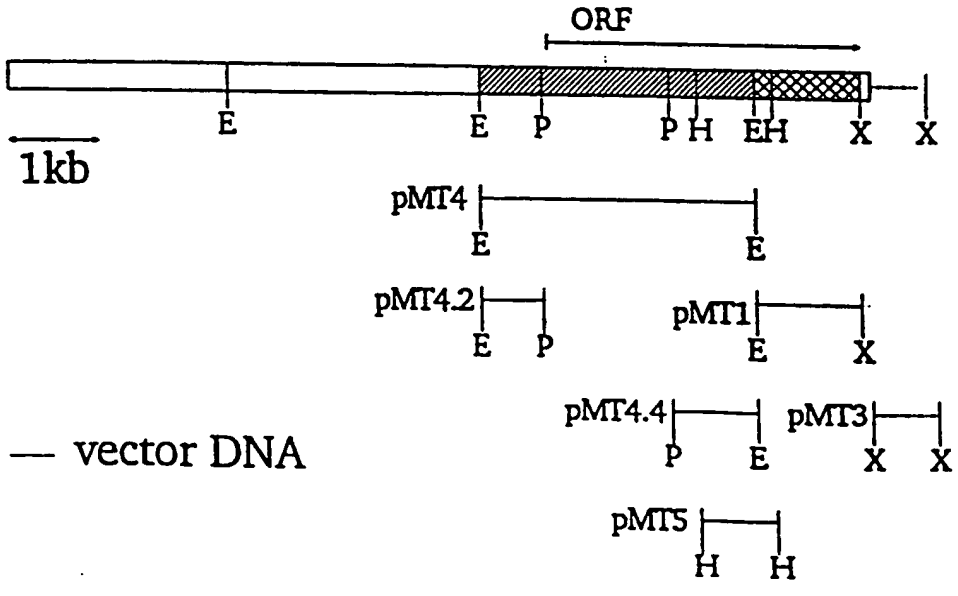
ORF
E
E P P H EH X
pMT4
E P P H E
pMT1
EH X
E = EcoRI P = PstI H = HindIII X = XhoI
DNA fragment recognized by oligo A
DNA fragment recognized by oligo B/C

Fig. 1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1kb
pMT4
E E
pMT4.2
E P
pMT1
E X
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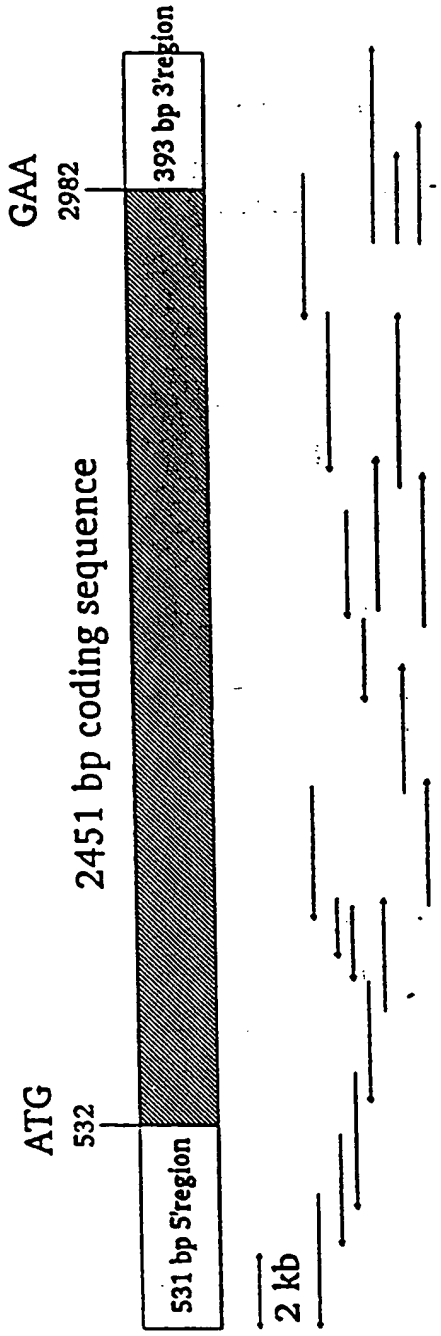
P E
pMT3
X X
— vector DNA
pMT5
H H
pMT4.1
P H
pMT4.3
P P

Fig. 2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2982
393 bp 3'region
2451 bp coding sequence
ATG
532
531 bp 5'region
2 kb

Fig. 3

Fig.4A

SEQ ID NO :1

TYPE OF SEQUENCE:	Nucleotides
LENGTH:	3375 base pairs
NUMBER OF STRANDS:	1
CONFIGURATION:	Linear
TYPE OF MOLECULE:	cDNA
ORIGIN :	Saccharomyces cerevisiae

```
   1  GCTTGTCTCC TGTTCATTCT ACGCTTGTCA TCCACACTGG CACCGGCAGC
  51  GGCGCTGCTC TGTGAGCTGT CTTTATCCAG CAGGCTCAAA TAGACTTGTT
 101  CTGCCTCATA TACTGTTTTT CATTAACCAG TCTCCTGGCC TCTTCTAATT
 151  TCGAACCTGG CAGAGACATG AAGTTCCTCG TTATAGATTA ATCACCAATT
 201  GTCCTCTTTC AGCGCTTCTG TTAGTTGGCC TTATTTAACG GATCTTTGCT
 251  TCAATTACGC TTTCATCCAA CATTTGCCAC CCTTGGAACC AGAAGAGAAT
 301  ATACATCATT CGGGGTTGCC CTGCCCATTT ATATCGTATA TTGTGATGAT
 351  ATACCTTTTT TTTTTTTTTC GCTGCGTTTT TTTTCTCGAC ACGTGTCGAA
 401  GAAGAGTTTG GCGTTTCTAA GCAGATCTTG ATTATTTTCG AGCAGCAAAA
 451  CAAGACAAAC AGGTGCATTG TTAAAGCGAG GTAGTATCAG AAGAGCCTAT
 501  CAAGAAACAG CTAACAGCTA CAAGCACGGT CATGTCGGAA GAGAAAACGT
 551  ACAAACGTGT AGAGCAGGAT GATCCCGTGC CCGAACTGGA TATCAAGCAG
 601  GGCCCCGTAA GACCCTTTAT TGTTACCGAT CCGAGTGCCG AATTGGCCTC
 651  GTTACGAACC ATGGTCACTC TTAAAGAGAA GCTGTTAGTG GCCTGTCTTG
 701  CTGTCTTTAC AGCGGTCATT AGATTGCATG GCTTGGCATG GCCTGACAGC
 751  GTGGTGTTTG ATGAAGTACA TTTCGGTGGG TTTGCCTCGC AATACATTAG
 801  GGGGACTTAC TTCATGGATG TGCATCCTCC TCTTGCAAAG ATGTTGTATG
 851  CTGGTGTGGC ATCGCTTGGT GGGTTCCAGG GTGATTTTGA CTTCGAAAAT
 901  ATTGGTGACA GCTTTCCATC TACGACGCCA TACGTGTTGA TGAGATTTTT
```

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1051	GCCGTTGAAA	ACTCGTACGT	CACTATTTCT	CGTTACATTC	TGTTGGACGC
1101	CCCATTGATG	TTTTTCATTG	CAGCTGCAGT	CTACTCTTTC	AAGAAATACG
1151	AAATGTACCC	TGCCAACTCG	CTCAATGCTT	ACAAGTCCTT	GCTTGCTACT
1201	GGTATTGCTC	TTGGTATGGC	ATCTTCATCC	AAATGGGTTG	GTCTTTTCAC
1251	GGTTACATGG	GTGGGTCTTT	TATGTATCTG	GAGACTATGG	TTCATGATTG
1301	GGGATTTGAC	TAAGTCTTCC	AAGTCCATCT	TCAAAGTAGC	ATTTGCCAAA
1351	TTGGCCTTCT	TGTTGGGTGT	GCCTTTTGCC	CTTTATCTGG	TCTTCTTTTA
1401	TATCCACTTC	CAATCATTAA	CTTTGGACGG	GGATGGCGCA	AGCTTCTTTT
1451	CGCCTGAATT	TAGATCTACA	CTAAAGAACA	ATAAGATCCC	CCAAAATGTC
1501	GTTGCTGATG	TCGGCATTGG	CTCCATTATC	AGCTTGCGTC	ATCTCTCTAC
1551	CATGGGCGGT	TATTTGCATT	CTCATTCACA	CAATTATCCA	GCTGGTTCGG
1601	AACAACAACA	AAGCACTTTA	TATCCTCACA	TGGATGCCAA	TAACGATTGG
1651	TTGTTGGAAC	TTTACAACGC	ACCCGGCGAA	TCTTTAACAA	CATTCCAAAA
1701	CCTAACCGAT	GGTACCAAGG	TCAGACTATT	CCACACTGTT	ACAAGATGTA
1751	GATTACACTC	TCATGACCAT	AAGCCACCCG	TTTCAGAAAG	CAGCGACTGG
1801	CAGAAGGAGG	TTTCTTGTTA	TGGTTACAGC	GGATTCGACG	GTGATGCTAA
1851	TGATGACTGG	GTTGTTGAGA	TTGATAAAAA	GAATTCTGCT	CCTGGAGTTG
1901	CCCAAGAACG	GGTCATAGCT	TTGGACACAA	AGTTTAGATT	GAGACATGCT
1951	ATGACAGGCT	GTTATTTGTT	TTCCCACGAA	GTCAAGTTGC	CAGCTTGGGG
2001	GTTCGAACAA	CAAGAAGTTA	CCTGTGCCTC	CTCCGGTAGA	CATGATTTAA
2051	CATTGTGGTA	CGTTGAGAAC	AACAGTAACC	CATTGTTACC	AGAAGATACC
2101	AAGCGTATTT	CCTATAAACC	TGCAAGCTTC	ATTTCTAAAT	TTATTGAATC
2151	CCATAAAAAG	ATGTGGCATA	TCAATAAAAA	TTTGGTCGAA	CCTCATGTTT
2201	ATGAATCACA	ACCAACTTCA	TGGCCATTCT	TGCTACGTGG	TATAAGTTAC
2251	TGGGGTGAAA	ATAACAGAAA	CGTCTATCTA	TTAGGTAATG	CGATCGTATG
2301	GTGGGCTGTC	ACCGCTTTCA	TCGGTATTTT	CGGATTGATT	GTTATCACTG

2351 AGCTGTTCTC GTGGCAGTTA GGTAAACCAA TTTTGAAGGA CTCCAAGGTT
2401 GTTAACTTCC ACGTTCAGGT TATTCACTAC TTATTGGGTT TTGCCGTCCA
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2501 TACCTGCTTA TTATTTCGGT ATTCTTGCCC TTGGCCACGC CTTGGACATA
2551 ATAGTTTCTT ATGTTTTCCG CAGCAAGAGA CAAATGGGCT ACGCGGTAGT
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2651 TTATTTACGG TACACCATGG ACTCAAGAAT TGTGTCAAAA ATCGCAGTGG
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2751 GTACAAAAAC CAAACCTTGA CTAAACGTGA ATCTCAACCT GCCGCCACTA
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3001 CCACATTTTT ATGTAAAGTC TTCTATAAGC TCTCGAGCGT ATAATTAAAA
3051 ACGAAAATAG ACAAAAAAAA CATCATGAAT AAAAAAAATG TCTTGAAGCT
3101 GACTATATTG TCCATCTGCG TTTAGAGACA CGTATTCTAT TTCGCTCAAA
3151 TAAGTATGAT CTGCAAGTAG TTTCAGTGGT ATTATCATTT CGCACCGTTT
3201 TTTTTCCAAG AACTCGTTTA CGTGCCGCGA AAAGTCTATC GAATAGGCAT
3251 TCGAGAACAA TAGAAAAGGA ACAGAAGCGT AGTACATATT ATGCATAGAC
3301 CCGTTTCTTT TCTTCTTTTT CGAAAATATT CTTATTGATT TAACAATTAA
3351 GCAGGTGTGT AAGATCAGAA CTGCA

SEQ ID NO: 2

TYPE OF SEQUENCE: Peptidic
LENGTH : 817 amino acids
PROPERTY: Dol-P-Man:protein(Ser/Thr)Mannosyltransferase
ORIGIN: Saccharomyces cerevisiae

```
   1  MSEEKTYKRV EQDDPVPELD IKQGPVRPFI VTDPSAELAS LRTMVTLKEK
  51  LLVACLAVFT AVIRLHGLAW PDSVVFDEVH FGGFASQYIR GTYFMDVHPP
 101  LAKMLYAGVA SLGGFQGDFD FENIGDSFPS TTPYVLMRFF SASLGALTVI
 151  LMYMTLRYSG VRMWVALMSA ICFAVENSYV TISRYILLDA PLMFFIAAAV
 201  YSFKKYEMYP ANSLNAYKSL LATGIALGMA SSSKWVGLFT VTWVGLLCIW
 251  RLWFMIGDLT KSSKSIFKVA FAKLAFLLGV PFALYLVFFY IHFQSLTLDG
 301  DGASFFSPEF RSTLKNNKIP QNVVADVGIG SIISLRHLST MGGYLHSHSH
 351  NYPAGSEQQQ STLYPHMDAN NDWLLELYNA PGESLTTFQN LTDGTKVRLF
 401  HTVTRCRLHS HDHKPPVSES SDWQKEVSCY GYSGFDGDAN DDWVVEIDKK
 451  NSAPGVAQER VIALDTKFRL RHAMTGCYLF SHEVKLPAWG FEQQEVTCAS
 501  SGRHDLTLWY VENNSNPLLP EDTKRISYKP ASFISKFIES HKKMWHINKN
 551  LVEPHVYESQ PTSWPFLLRG ISYWGENNRN VYLLGNAIVW WAVTAFIGIF
 601  GLIVITELFS WQLGKPILKD SKVVNFHVQV IHYLLGFAVH YAPSFLMQRQ
 651  MFLHHYLPAY YFGILALGHA LDIIVSYVFR SKRQMGYAVV ITFLAASVYF
 701  FKSFSPIIYG TPWTQELCQK SQWLSGWDYN CNTYFSSLEE YKNQTLTKRE
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 801  EEEGANILKV EKRAVLE
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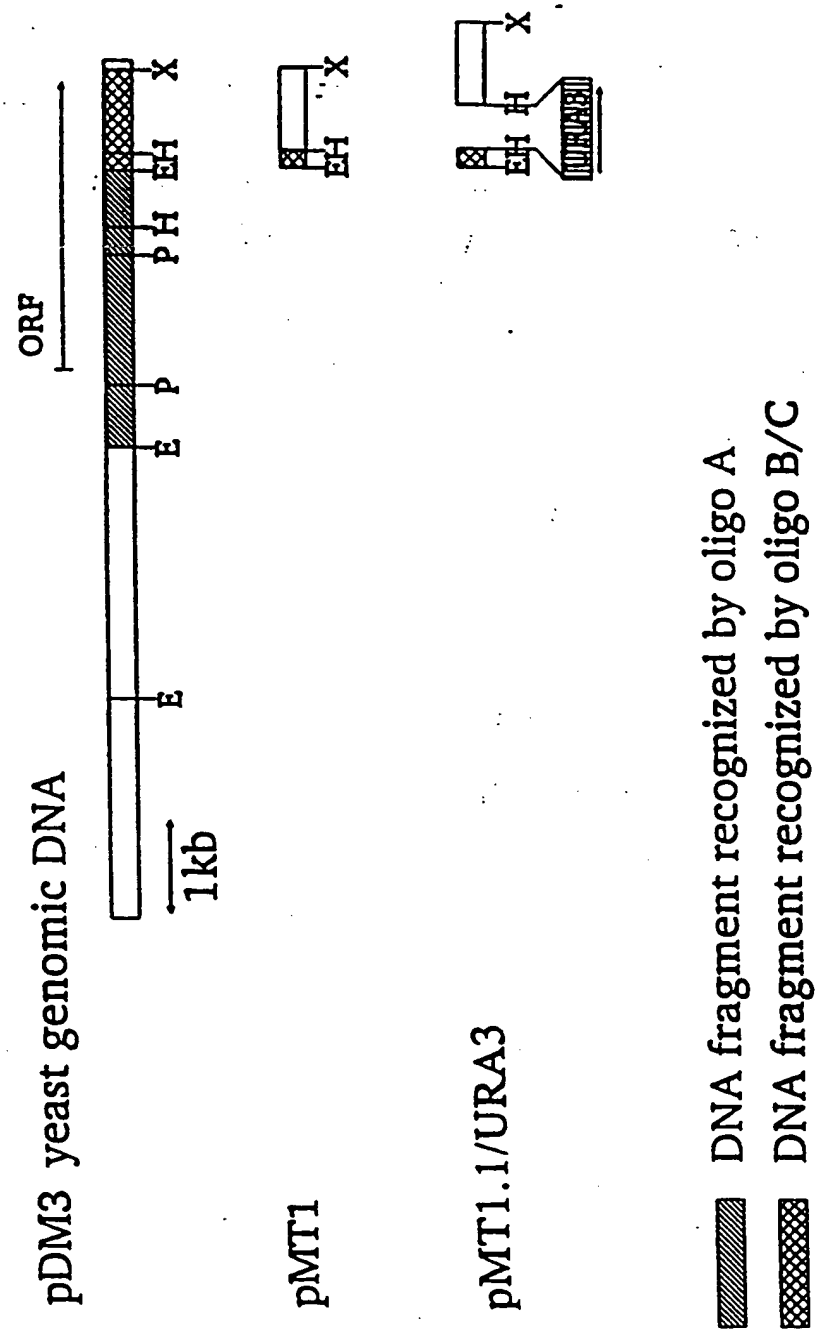
Fig.4B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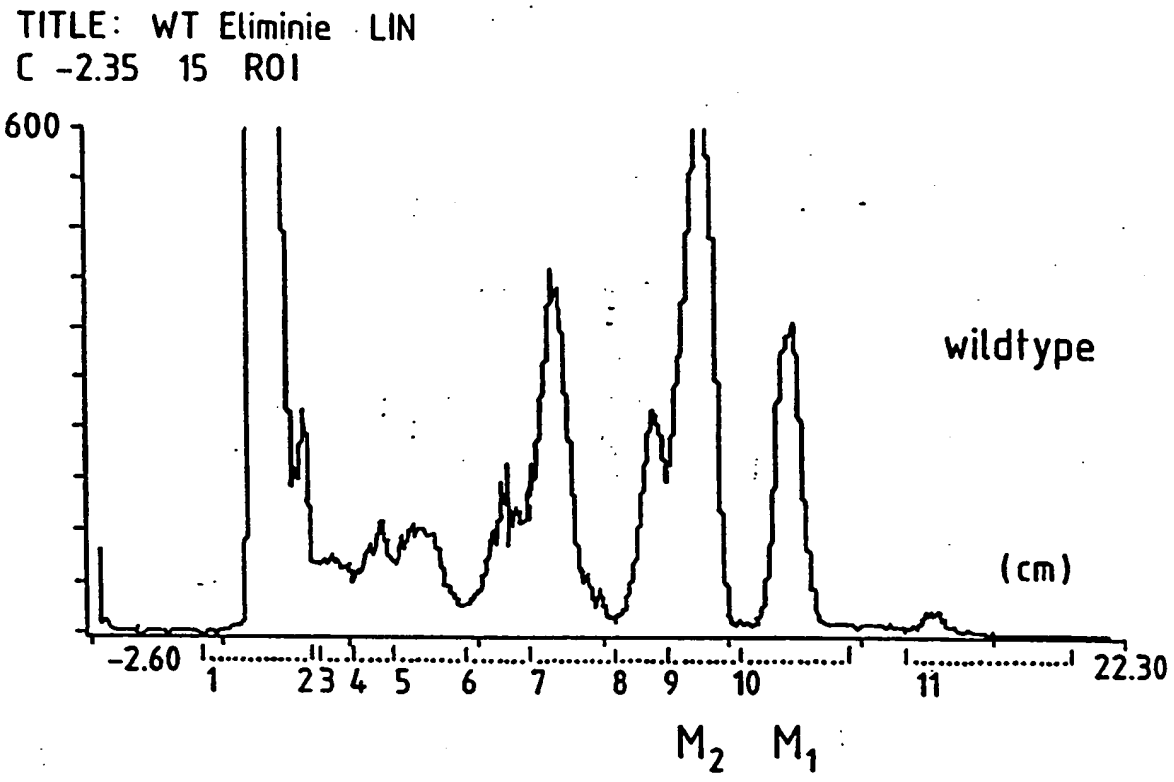
DNA fragment recognized by oligo A
DNA fragment recognized by oligo B/C

Fig. 5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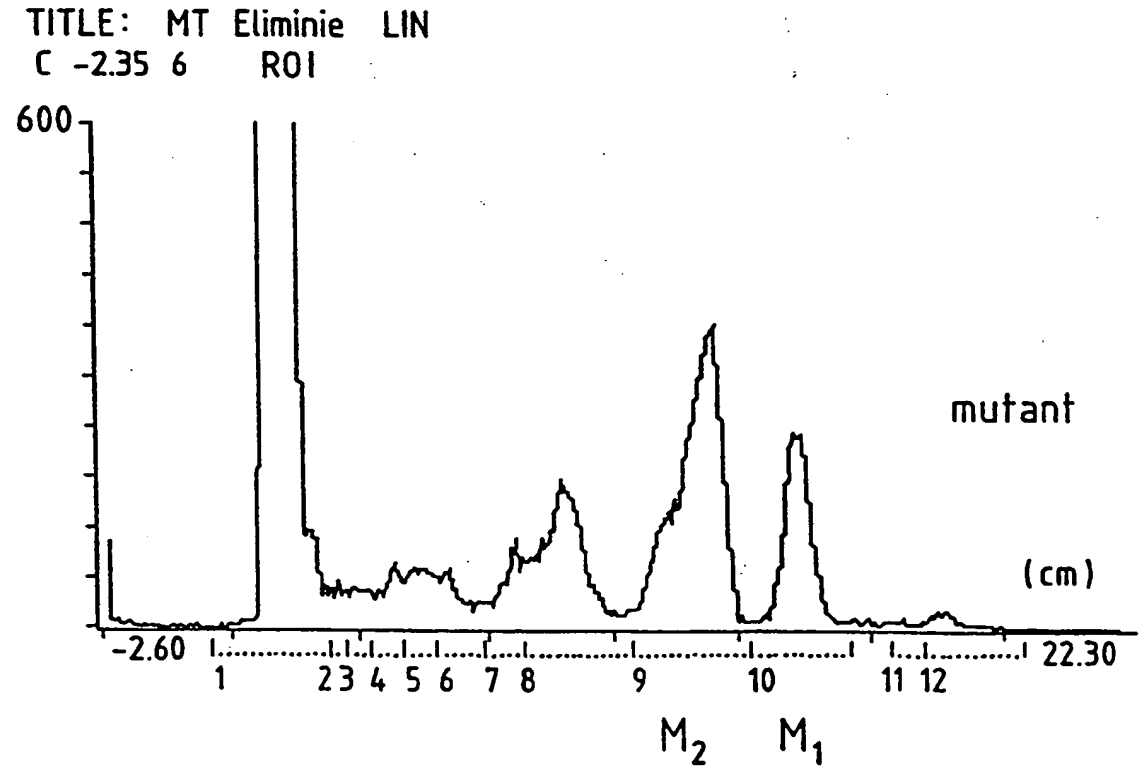
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(cm)
-2.60
1
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11 12
22.30
M2 M1

Fig. 6

SEQ ID No. 3

Sequence Type:	DNA
Sequence Length:	126 base pairs
Topology:	linear
Strandedness:	single
Feature:	part of the K. lactis Dol-P-Man:protein (Ser/Thr) mannosyltransferase encoding gene (open reading frame)
Origin:	Kluyveromyces lactis

```
  1 TCTGCTCCTG GCGNAGCTCA AGTATNCGTT AAGGCTTTGG ACACTAAATT
 51 CAGATTGAGA CATGCTATGA CTGGTTGTAG TATCTCACAT GAAGTCAAAT
101 TACCAAAATG GGGCTTCGAA CAACAG
```

Fig.7

(A)

position 1885 of SEQ. ID N° 1

```
TCTGCTCCT  GGAGTTGCC  CAAGAACGG  GTCATAGCT  TTGGACACA  AAGTTTAGA
TCTGCTCCT  GGCG?AGCT  CAAGTAT?C  GTTAAGGCT  TTGGACACT  AAATTCAGA

TTGAGACAT  GCTATGACA  GGCTGTTAT  TTGTTTTCC  CACGAAGTC  AAGTTGCCA
TTGAGACAT  GCTATGACT  GGTTGTAGT  ATC---TCA  CATGAAGTC  AAATTACCA

GCTTGGGGG  TTCGAACAA  CAA
AAATGGGGC  TTCGAACAA  CAG
```

(B)

position 452 of SEQ. ID N° 2

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SAPGVAQER  VIALDTKFR  LRHAMTGCY  LFSHEVKLP  AWGFEQQ
SAPG?AQV?  VKALDTKFR  LRHAMTGCS  I-SHEVKLP  KWGFEQQ
```

Fig.8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 93/02179

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/54 C12N9/10 C12N15/81 C12N1/15 C12N1/19
C12P21/00 C12Q1/68 //(C12N1/19,C12R1:865)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C12P C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EUR. J. BIOCHEM. vol. 196, no. 1 , February 1991 , SPRINGER VERLAG, BERLIN, BRD; pages 185 - 190 S. STRAHL-BOLSINGER AND W. TANNER 'Protein O-glycosylation in Saccharomyces cerevisae' cited in the application see page 189, right column, line 26 - page 190, left column, line 2 ---	1-30
Y	US,A,4 929 553 (BUSSEY ET AL.) 29 May 1990 see column 3, line 60 - column 4, line 8 see column 6, line 6 - line 31 ---	1-30
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 December 1993	18 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FEBS LETTERS vol. 248, no. 1,2 , 8 May 1989 , ELSEVIER PUBLISHERS, AMSTERDAM, NL; pages 111 - 114 K. HARD ET AL. 'O-mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in Saccharomyces cerevisiae' cited in the application the whole document	1-30
A	METHODS IN ENZYMOLOGY vol. 194 , 1991 , ACADEMIC PRESS, INC., NEW YORK, US; pages 281 - 301 R. ROTHSTEIN 'Targeting, disruption, replacement, and allele rescue: Integrative DNA transformation in yeast' cited in the application see page 290, line 9 - page 298, line 17	1-30
A	BIO/TECHNOLOGY vol. 8, no. 2 , February 1990 , NATURE AMERICA, INC., NEW YORK, US; pages 135 - 139 J.A. VAN DEN BERG ET AL. 'Kluveromyces as a host for heterologous gene expression: Expression and secretion of prochymosin' cited in the application the whole document	1-30